THE DEVELOPMENT OF TESTING PROCEDURES TO DETECT BOVINE

SERUM IGM TO BLUETONGUE VIRUS

by

LISA MARIE BURDETT

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Major Professor

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I would like to thank Dr. Phillips for making this possible. I would also like to thank Dr. Leudke for allowing us to use his serum samples. Thanks to Sylvia Osborne for all her help. To all my friends and family who helped me through all this--thank you.

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Bluetongue is an arthropod borne viral disease. The virus is an orbivirus in the reoviridae family. It is antigenically related to African Horse Sickness and Epizootic Hemorrhagic Fever. Worldwide, there are currently 23 serotypes recognized. In the United States, there are currently five serotypes that have been identified. The incidence is the lowest in the New England states and the highest in the South and Southwestern states.

Bluetongue virus has long been recognized as an important viral disease of sheep. This disease, however, has cost the United States cattle industry an estimated 30 million dollars in loss of cattle, semen and embryo exports.² Another source has estimated that as much as 24 million dollars a year is lost as revenue due to the ban of semen export to the United Kingdom, Australia and New Zealand.³ This demonstrates the need for a viable and reliable test to determine the current infectious status of cattle.

Bluetongue virus was first isolated in California in 1952. Two possible introductions of the disease to the United States are considered to be most likely. One was the importation of Black-bellied sheep in 1904 to Maryland from Barbados and the other was the importation of

Afrikander cattle to Texas in 1931. The disease had been described as early as the 1940s in cattle and sheep as sore muzzle disease. 1

Bluetongue disease is usually asymptomatic in cattle with only 5% of infected cattle showing clinical signs. 1
When the symptoms do occur, there is usually a transient febrile stage, excessive salivation, localized inflammation with some necrosis of the buccal mucosa, lesions on the udder, coronitis, stiffness of the gait, lameness and/or the lack of condition. In the pregnant animal, abortions or congenital defects or death of the newborn may occur. Partial differential diagnoses may include Foot and Mouth Disease, IBR, BVD, vesicular stomatitis, or mycotic stomatitis.

Bluetongue virus replicates in vascular endothelial cells. Eventually, vascular occlusion, stasis and exudation occur. This compromises the vessels leading to hypoxia of the overlying epithelium with secondary development of lesions. 4

Three species of midges (<u>Culicoides</u>) are known vectors of bluetongue. All ruminants appear to be susceptible.

Because the disease is difficult to detect in cattle, widespread disease, as well as severe economic impact may occur before the etiological agent can be discovered. One

outbreak of bluetongue in Mississippi and four surrounding states cost 12 million dollars.

The impact on ruminant wildlife is considerable. In addition to direct losses, testing of livestock represents considerable cost.

Canada is one of the bluetongue free countries that will allow the importation of cattle from countries where bluetongue is endemic. Canada requires two Agar Gel Immunodiffusion (AGID) tests run within a specified number of days (the second test no less than 30 days and no more than 90 days after the first test, but within 30 days prior to importation to canada.⁵) The initial test must be on the entire herd, and the second test is required on only those animals that will be imported. Other Bluetongue free countries, such as Great Britain, prohibit the importation of any cattle from the United States.³,⁵

Currently, three virus isolation techniques are in use. All are considered insensitive methods. The wild strain of the virus needs to be "adapted" for cell culture by passage through embryonating chicken eggs by intravenous (IV) inoculation. Tissue cell cultures used are Panama and African Green Monkeys vero cells, although Aedes albopictus C3/C6, a mosquito cell line, is considered to be more sensitive. The most sensitive method is by inoculating sheep, but it is also quite expensive.

Serological tests currently in use have inherent problems. The Compliment Fixation (CF) test is a group specific test as is the Agar Gel Immunodiffusion (AGID) test. The AGID test is thought to be more sensitive. 3 The AGID indiscriminantly checks for antibodies against bluetonque, whether they are IgG or IgM antibodies. Up to 43% of cattle positive for bluetonque by virus isolation were negative on the AGID test. 6 Epidemiologically, in the western United States, it has been shown that cattle were demonstrated to be free of bluetonque virus regardless of the serological status in the winter months. 4 No single test is adequate at this time to determine the disease status of the animal. Therefore, a test that will determine IgM levels specifically against bluetonque would be considered more sensitive in determining the infectious status. A more sensitive and more reliable test may allow some cattle currently rejected to be considered suitable for export.

IgM is found in the serum in the second highest concentration of the immunoglobulins. The molecular weight of the IgM molecule is 900k daltons. Due to its size, IgM is mainly confined to the blood vascular system. The also carries a u antigenic determinant. Five 7s monomers are linked by disulfide bonds to create a 19s pentamer. A cysteine rich J chain links two of the subunits.

In the primary immune response, IgM is the major antibody produced. It is considered to be more efficient on a molar basis than IgG. IgM levels are usually highest in an active infection. IgM first appears in the blood 7 to 10 days after primary infection, and maximum titers usually occur in 2 to 3 weeks. IgM levels are usually undetectable three months after an infection. A test to determine serum IgM levels should therefore be an accurate method of determining the early infectious status of the animal.

MATERIALS AND METHODS

Viral Preparation Bluetongue virus, serotype 17, was grown in Aedes albopictus C6/36 cell line with MEM medium containing 10% inactivated fetal calf serum at 33° C. After two passages in the mosquito cell line the virus was grown in baby hamster kidney cell line (BHK 21) and a vero cell line. Infected flasks were kept at 35°C or 37° C and cells harvested at intervals, to determine optimal concentration of viral particles. The BHK 21 cells yielded the highest viral titer at 37°C. Virus preparation was by a variation of the procedure used by Anderson. At 36 hours post infection, cells were freed by tapping the flasks and harvested. The media and cellular debris were centrifuged at 1,000 x g for 10 minutes at 4°C.

a lysis buffer (1/10 volume) and sonicated on ice four times with a Sonic Dismembrator 300 (Artex) in 30 second bursts at 35% relative output. The sample was then centrifuged at 10,000 x g for 10 minutes at 4° C. The supernatant was saved. This step was repeated two more times. The average titer of the virus was 1x $10^{-5}/.025$ ml.

Serum Samples. Eighty-eight test sera were obtained from three sources. Twenty-two samples from experimentally infected cattle were obtained from Dr. Leudke in Wyoming. (Group One). Group Two consisted of 30 serum samples from two years positive sera from the bluetongue check tests supplied by National Veterinary Service Laboratory (NVSL). Group Three contained thirty-six samples which were obtained from the Veterinary Diagnostic Laboratory at Kansas State University. These bovine samples were all positive on the AGID test.

Sample Preparation. All three group sera were eluted by centrifugation through a polyacrylamide liquid chromatography gel, Bio-Gel P-200, to remove proteins with a molecular weight greater than 200,000 daltons as used by Pretzman, et al. ¹⁰ All samples at the 1:30 dilution after the P-200 gel were also treated with 0.015M 2-mercaptoethanol. ¹¹ Fifty-six samples were also heat inactivated at 65° C for 15 minutes. ⁷

Serum Neutralization Tests All samples were checked

for neutralizing antibodies using vero cell cultures. Virus was diluted 1/10 from the sonicated stock giving a titer of 1×10^{-4} as the working dilution. Cultures were examined for cytopathic effects after four days. Samples were also screened by the serum neutralization test at the 1:6 dilution after elution through a P-200 gel and at the 1:30 dilution after 2-mercaptoethanol treatment.

Indirect Immunofluorescence Test All samples were tested at the 1:30 and 1:60 dilution. One tenth ml of RHK 21 or vero cells at approximately 250,000 cells/ml were planted in 10 well slides with 0.025 ml of bluetonque virus, serotype 17, at 1 x 10^{-3} titer. Slides were harvested and fixed in acetone after 36 hours and stored at -70° C until used. Serum samples were diluted 1:30 and 1:60 then 0.025 ml placed in each well and incubated at 37°C for 30 minutes. Slides were next washed twice in 0.01M PBS for five minutes and once in distilled water for five minutes. Slides were dried and 0.025 ml of fluorescein conjugated antisera was placed on the wells. Slides were incubated with conjugate for 30 minutes at 37°C. then washed and dried as before. Anti-IgM, mu specific (Kirkegaard & Perry Laboratories, Inc.) and anti-IgG, gamma specific fluorescein-labeled antibodies were used at 1:40 dilution. Coverslips were applied with glycerol buffered mounting media, then slides were examined by fluorescent

microscopy.

DATA

SEROLOGICAL TESTS ON UNTREATED SERA

Agar Gel Immunodiffusion

All samples tested were positive by AGID for antibodies against bluetongue before column chromatography. All samples were negative for antibodies to bluetongue on the AGID after the column. The P-200 column eliminated the majority of the precipitating antibodies; IgG is one of the major precipitating immunoglobulins.

Serum Neutralization

Group One sera were all positive on the AGID test. All of the sera also had titers to bluetongue virus on the serum neutralization test. Leudke had used bluetongue virus serotype 11 to infect the cattle. All of our tests used serotype 17. Since these samples contained antibodies to serotype 17 and were from cattle known to have been infected with serotype 11, there is cross reactivity between these two serotypes. After eighteen months, cattle still had detectable antibodies to the bluetongue virus. Table 1.

In Group Two, serum neutralizing antibody titers ranged from <1:8 to 1:256. Figure 1.

In Group Three, thirty-six samples were checked for serum neutralizing antibodies against bluetongue. Serum

neutralizing antibody titers ranged from <1:2 to >1:256. See Figure 2.

Indirect Immunofluorescence Antibody Test

In Group One, the indirect immunofluorescence antibody (IFA) test demonstrated that three samples (14%) at the 1:60 dilution before elution of the samples through the P-200 column were positive when checked for IgM. Seven (33%) of the samples were weakly positive and the rest (57%) of the samples were negative.

In Group Two, seven samples (23%) were positive for IgM specific against bluetongue. Twenty-two (73%) were negative and one sample was weakly positive.

In Group Three, all samples were checked for IgM at the 1:60 dilution before elution through the P-200 column. Sixteen samples (44%) demonstrated IgM specific against bluetongue. Sixteen samples (44%) were negative and four samples were weakly positive.

Radial Immunodiffusion test

Seventeen samples were checked for serum IgM concentration by utilizing a single radial immunodiffusion kit supplied by V.M.R.D.Inc. 12 Samples were taken from 50 days after insect transmission to 550 days after insect transmission. All samples checked had IgM levels ranging from 125 mg/dl to 250 mg/dl.

SEROLOGICAL TESTS ON SERA ELUTED THROUGH P-200 COLUMN Serum Neutralization

The SN test proved to be inconclusive on the samples screened at the 1:6 dilution after the P-200 column.

Thirteen samples were shown to be toxic to the cells. Of those samples in Group One that were not toxic, eight demonstrated the presence of neutralizing antibodies while only one lacked neutralizing antibodies at the 1:6 dilution. In Group Two, twenty-seven of the samples (90%) were toxic at the 1:6 dilution. Two samples were negative and one sample was not tested. Group Three samples were eluted through a P-200 column and checked at the 1:6 dilution for serum neutralizing antibodies against bluetongue. Eighteen samples (50%) were toxic. Four samples (11%) demonstrated no serum neutralizing antibodies while eight samples (22%) had neutralizing antibodies to bluetongue. Six samples were not tested.

Serum Neutralization-Treated with 2-mercaptoethanol.

All samples were screened at the 1:30 dilution after treatment with 2-mercaptoethanol. In Group One, five samples demonstrated antibodies, while the remaining samples were negative. In Group Two, fourteen samples (47%) demonstrated no serum neutralizing antibodies at this dilution. Fifteen samples (50%) did demonstrate serum neutralizing antibodies. One sample was contaminated and a

serum neutralization titer could not be evaluated.

In Group Three, five samples (14%) were either toxic to the cell culture, or contaminated. Thirteen samples (36%) had serum neutralizing antibodies against bluetongue while eighteen samples (50%) were negative.

Indirect Immunofluorescence Antibody Test

In Group One, samples eluted through the column, all samples were checked at dilutions of 1:30 and 1:60 for the presence of IgM specific to bluetongue virus. At the 1:30 dilution, eleven (50%) samples were negative, ten (45%) of the samples were positive and one sample was weakly positive. At the 1:60 dilution, three samples were positive. Seventeen samples were negative and two samples were weakly positive.

In Group Two, twenty-four samples (80%) demonstrated IgM antibodies specific against bluetongue virus at the 1:30 dilution. Six samples (20%) demonstrated no IgM antibodies. At the 1:60 dilution, eleven samples (37%) demonstrated specific antibodies against bluetongue virus, and nineteen samples (63%) were negative.

In Group Three, all samples were checked at 1:30 and 1:60 dilutions on the indirect immunofluorescent antibody test for IgM specific against bluetongue. At the 1:30 dilution, twenty-three samples (64%) demonstrated IgM specific against bluetongue. Six samples (17%) were

negative and seven samples (19%) were weakly positive. At the 1:60 dilution, eighteen samples (50%) demonstrated IgM specific against bluetongue. Fourteen samples (39%) were negative, while five samples (14%) were weakly positive. Indirect Immunofluorescent Antibody Test- sera treated with 2-mercaptoethanol.

In Group One, the samples demonstrated more weak positives at the 1:30 dilution after the treatment with 2-mercaptoethanol. Eight samples (36%) were weakly positive. Four samples (18%) demonstrated IgM antibodies specific to bluetongue, and ten samples (45%) were negative.

In Group Two, the samples were all treated with 2-mercaptoethanol at the 1:30 dilution. Nine samples (30%) demonstrated no IgM antibodies specific against bluetongue virus. Nineteen samples (63%) did demonstrate antibodies specific against bluetongue, while two samples (7%) were weakly positive.

In Group Three, after treatment with 2-mercaptoethanol at the 1:30 dilution, eight samples (20%) were negative, eleven samples (34%) were weakly positive. Seventeen samples (46%) demonstrated IgM specific against bluetongue. Indirect Immunofluorescent Antibody Test-samples heat inactivated.

Samples in Group One were not heat inactivated.

In Group Two, heat inactivation at the 1:30 dilution

after elution through a P-200 column yielded fifteen samples (50%) demonstrating IgM antibodies specific against bluetongue virus. Eleven samples (37%) demonstrated no antibodies, and four samples were not tested.

The samples in Group Three were heat inactivated at the 1:30 dilution. Fifteen samples (42%) demonstrated IgM specific against bluetongue. Fifteen samples (42%) were negative and six samples were not tested.

Indirect Immunofluorescent Antibody Test-testing for the

Indirect Immunofluorescent Antibody Test-testing for the presence of IgG.

Samples in Group One were checked for the presence of IgG antibodies to the bluetongue virus after the column. Nineteen samples (86%) demonstrated IgG antibodies while only three were negative.

All samples in Group Two were also checked after elution at the 1:30 dilution for the presence for IgG specific against bluetongue virus. Twenty-three samples (77%) demonstrated IgG antibodies specific against bluetongue. Three samples (10%) were negative and four sample (13%) were weakly positive.

In Group Three, all samples were checked for the presence of IgG specific against bluetongue. Eighteen samples (50%) demonstrated IgG antibodies against bluetongue. Sixteen samples (44%) were negative and two samples were weakly positive.

DISCUSSION

In Group One, one animal retained a serum neutralizing titer against bluetonque virus after 611 days. Fifty days post infection, IqM was detectable on the indirect immunofluorescent antibody test. Of those animals experimentally infected (Group One), there were samples checked before the P-200 column which were negative and after the column were positive, suggesting that there was competitive adsorption for bluetongue infected sites between IqG and IqM. Once the sera had been eluted through the column, eliminating the majority of the IgG, the IgM molecule was no longer competing with other molecules for the antigen sites. Four samples tested weakly positive before the column and negative after elution for serum IqM to bluetongue. Fluorescein tagged antibodies may have reacted with IgG dimers, or slides prepared at different times may have been slightly different in sensitivity may account for the results obtained.

The majority (92%) of the samples in Group One that had titers of 1:8 on the serum neutralization test had either negative results or weakly positive results on the indirect immunofluorescent antibody test when assayed for IgM specific against bluetongue virus before the column.

The majority of the samples did have IgG antibodies

specific against bluetongue virus after the column. Only three were negative. Nine samples had IgG antibodies, with no IgM antibodies detectable. These results show that the fluorescein tagged anti-IgM (mu determinant) is specific, as is the fluorescein tagged anti-IgG (gamma specific).

Treatment with 2-mercaptoethanol resulted in ambiguous results. The treatment was gentle, to prevent disruption of internal disulfide bonds. Positive results occurred post 2-mercaptoethanol treatment. These results could be attributed to the gentle treatment with a low concentration of 2-mercaptoethanol, where not all of the disulfide bonds were disrupted. Another possible explanation is the reorganization of disulfide bonds.

The remaining sixty-six samples, Groups Two and Three, had serum neutralizing antibody titers ranging from <1:2 to >1:256 before column chromatography. All samples were positive on AGID for bluetongue antibodies before elution through the P-200 column and negative on the AGID for bluetongue antibodies after the P-200 column. Samples showed no known relationship between the serum neutralizing antibody titers and the results on the indirect fluorescent antibody test for IgM. Two samples which demonstrated no serum neutralizing antibodies were positive before elution through the P-200 column at the 1:60 dilution for IgM

antibodies to bluetongue virus, confirming that the IFA test is a sensitive test.

The 2-mercaptoethanol treatment resulted in more weakly positive results, suggesting that the treatment was too gentle and did not disrupt all of the disulfide bonds in the IgM molecule.

Heat inactivation at 65° C for 15 minutes was also used to disrupt the disulfide bonds. In this treatment, the ambiguity was not as apparent. Several instances demonstrated that the bonds were disrupted resulting in negative results on the indirect immunofluorescent antibody test.

The serum samples submitted to the Diagnostic
Laboratory included seventeen negative samples at the 1:30
dilution after elution through the P-200 column for IgG
specific antibodies. The check tests only represented 2
negative samples of the 30 samples checked. This suggests
that the level of the IgG in the check test serum samples
may have been higher. In most cases, samples submitted to
the Diagnostic Laboratory are from sick animals. IgM
levels may have been elevated due to illness.

Since IgM levels remained detectable up through 611 days post infection, the development of a test to detect IgM levels to bluetongue virus as an early indicator of an early infection would be of limited value.

TABLE 1.Serum neutralization test results for seronegative cattle infected with bluetonque serotype 11 by insect transmission.

SAMPLE NO.	ANIMAL days post transmission	TITER	AC ^a 1:6	2-ME ^b 1:30 AC	
01	A-450 ^C	1:32	т	P	
02	A-563	1:16	T	P	
03	A-611	1:16	P	P	
04	B-050	1:64	T	P	
05	B-550	1:8	T	N	
06	C-450	1:8	P	N	
07	C-500	1:8	T	N	
08	D-350	1:8	T	N	
09	D-500	1:8	P	N	
10	E-300	1:8	P	N	
11	E-400	1:8	P	N	
12	F-350	1:8	T	N	
13	F-400	1:8	T	N	
14	G-250	1:16	P	N	
15	G-300	1:16	T	N	
16	H-200	1:16	ND	P	
17	H-300	1:8	P	N	
18	I-150	1:8	T	N	
19	I-250	1:16	T	N	
20	J-100	1:16	T	N	
21	J-200	1:4	T	N	
22	J-239	1:8	N	N	

a: After elution through P-200 Column

b: Samples treated with 2-mercaptoethanol-after the column.

c: The numbers represent the days after insect

transmission.

T: Toxic

P: Positive-antibodies demonstrated.

N: Negative-no antibodies demonstrated.

ND: Not Done

TABLE 2.Indirect immunofluorescent antibody test^a results on seronegative cattle infected with bluetonque virus serotype 11 by insect transmission.

SAMPLE NO.	ANIMAL days pos infectio		AC ^C 1:30	AC 1:60	2-ME ^d 1:30	anti-IgG AC 1:30	
01	A-450 ^e	Nf	Pa	N	WPh	P	
02	A-563	P	P	P	P	P	
03	A-611	P	P	P	P	P	
04	B-050	WP	P	P	WP	P	
05	B-550	P	P	N	N	N	
06	C-450	N	P	N	P	P	
07	C-500	N	N	N	N	P	
08	D-350	N	P	N	WP	P	
09	D-500	N	N	N	WP	P	
10	E-300	N	P	N	P	P	
11	E-400	WP	N	N	N	P	
12	F-350	WP	N	N	N	P	
13	F-400	N	N	N	N	P	
14	G-250	N	P	WP	N	P	
15	G-300	N	N	N	N	P	
16	H-200	WP	N	N	WP	N	
17	H-300	N	N	N	N	N	
18	I-150	N	N	N	WP	P	
19	I-250	N	N	N	N	P	
20	J-100	WP	WP	N	WP	P	
21	J-200	WP	N	N	WP	P	
22	J-239	WP	P	WP	N	P	

a: All tests but the last column were checked for IgM specific against bluetongue--the last column was checked for IgG.

b: Before elution through P-200 Column.

c: After elution through P-200 Column.

d: Samples treated with 2-mercaptoethanol-after the column.

e: The numbers represent the days after insect transmission.

N: Negative-no antibodies demonstrated.

P: Positive-antibodies demonstrated.

WP: Weak Positive.

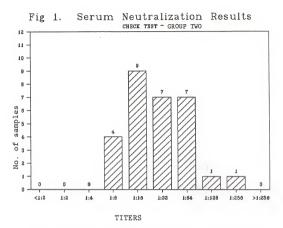


Fig. 1. Results on serum neutralization test on positive NVSL check sera before the column.

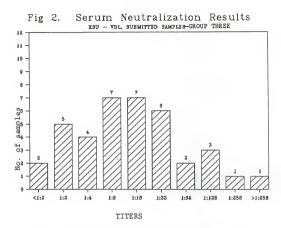


Fig. 2. Results on serum neutralization test on samples submitted to the diagnostic lab-Group three-before the column.



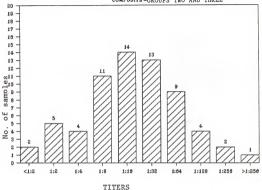


Fig. 3. Results of the serum neutralization test on both $\hbox{Groups Two and Three before the column.}$

Fig 4. IFAT Results

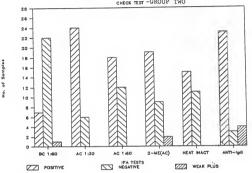


Figure 4.

- BC 1:60- Samples checked at the 1:60 dilution before elution through the column for IgM antibodies
- AC 1:30- Samples checked at the 1:30 dilution after elution through the column for IgM antibodies.
- AC 1:60- Samples checked at the 1:60 dilution after elution through the column for IgM antibodies.
- 2-ME 1:30- Samples checked at the 1:30 dilution after the column, after treatment with 2-mercaptoethanol for IgM antibodies.
- Heat Inact- Samples checked at the 1:30 dilution after elution, and after heat inactivation at 65 C for 15 minutes for IgM antibodies.
- Anti-IgG- Samples checked at the 1:30 dilution after elution through the column for IgG antibodies.

Fig 5. IFAT Results

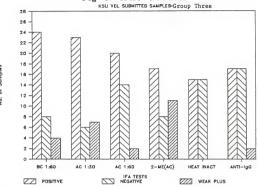


Figure 5.

- BC 1:60- Samples checked at the 1:60 dilution before elution through the column fro IgM antibodies.
- AC 1:30- Samples checked at the 1:30 dilution after elution through the column for IgM antibodies.
- AC 1:60- Samples checked at the 1:60 dilution after elution through the column for IgM antibodies.
- 2-ME 1:30- Samples checked at the 1:30 dilution after the column, after treatment with 2-mercaptoethanol for IRM antibodies.
- Heat Inact- Samples checked at the 1:30 dilution after elution, and after heat inactivation at 65°C for 15 minutes for IRM antibodies.
- Anti-IgG- Samples checked at the 1:30 dilution after elution through the column for IgG antibodies.

Fig 6. IFAT Results

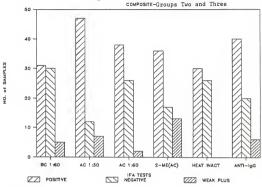


Figure 6.

- BC 1:60- Samples checked at the 1:60 dilution before elution through the column for IgM antibodies.
- AC 1:30- Samples checked at the 1:30 dilution after elution through the column for IgM antibodies.
- AC 1:60- Samples checked at the 1:60 dilution after elution through the column for IgM antibodies.
- 2-ME 1:30- Samples checked at the 1:30 dilution after the column, after treatment with 2-mercaptoethanol for IgM antibodies.
- Heat Inact- Samples checked at the 1:30 dilution after elution, and after heat inactivation at 65°C for 15 minutes for IgM antibodies.
- Anti-IgG- Samples checked at the 1:30 dilution after elution through the column for IgG antibodies.

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THE DEVELOPMENT OF TESTING PROCEDURES TO DETECT BOVINE SERUM IGM TO BLUETONGUE VIRUS

by

LISA MARIE BURDETT

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Serum samples from experimentally infected bovine, serum from the National Veterinary Service Laboratory and samples submitted to the Diagnostic Laboratory at Kansas State University were assayed by the serum neutralization test, the indirect immunofluorescent antibody test(IFAT), and the agar gel immunodiffusion test. Samples were tested conventionally, treated with 2-mercaptoethanol, and heat inactivated. Samples were also subjected to spun column chromatography, to concentrate the serum IgM, then checked on the IFAT for specific antibodies to bluetongue virus.

Experimentally infected cattle were exposed to bluetongue virus serotype 11; our tests used bluetongue serotype 17. There was cross-reactivity between the serotypes. We found serum IgM to BTV was detectable from 50 days through 611 days after experimentally infected with BTV. Using the techniques applied, the development of a bovine serum IgM test for bluetongue virus is of little value in detecting an early infection to bluetongue.